A multi-residue TLC screening procedure for anabolic oestrogens and detection of oestradiol, DES or zeranol in chicken muscle tissue extracts

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A multi-residue HETLC (High Efficiency Thin Layer Chromatography) screening procedure for 17β-oestradiol, diethylstilboesterol (DES), zearalanol (zeranol), zearalenone and their metabolites oestrone, zearalanone, and zearalenol is described. The anabolic oestrogens were analyzed on HETLC plates coated with silica gel and were developed in methylene chloride:methanol: 2-propanol (97 : 1 : 2 v/v). The spots were visualized by exposure to iodine vapours and subsequently sprayed with 1% starch solution. Analysis of standards by HETLC at 4 °C as a seven-component mixture showed six discrete bands with mean R_fs of 0·37 (oestrone), 0·35 (zearalanone and zearalenone), 0·26 (t-DES), 0·23 (oestradiol), 0·17 (zearalenol and zearalanol), and 0·15 (c-DES). Chicken muscle tissues (1, 2·5, or 5 g) were extracted with 95% acetone. Extracts were then fortified with 50–250 ng each of the anabolic oestrogens, purified in alumina and ion-exchange columns and analyzed by HETLC. Oestradiol, zeranol or DES in fortified tissue extracts were clearly detected when an equivalent of 4 ng were analyzed by HETLC after purification in alumina and ion-exchange columns. The intensity of their bands suggested near quantitative recovery when compared to intensity of bands of known amounts of standards. The described extraction, purification, and TLC procedures can be used to screen these oestrogens at low ppb amounts in chicken muscle tissues and should be applicable to screen tissues of cattle and sheep.

Introduction

Anabolic oestrogens are used in animal production (cattle and sheep) to enhance rapid growth and to improve feed efficiency resulting in economic benefits to farmers and consumers as well. Their use in poultry (turkey and chickens) improves carcass quality. In the United States, 17β-oestradiol, its ester 17β-oestradiol benzoate, and zearalanol (zeranol) are permitted for use in cattle and sheep (Herrick 1984), while 17β-oestradiol monopalmitate is allowed in chicken roasters (Code of Federal Regulations 1986). Oestradiol and its metabolite, oestrone, are endogenous hormones in humans and animals. Zearalanol is industrially produced from zearalenone, a mycotoxin elicited by *Fusarium* moulds (Baldwin *et al.* 1983). Diethylstilboesterol (DES), a synthetic hormone previously used in cattle and sheep production, is banned in the United States, Canada and EEC member countries. Improper use of these hormones or lack of sufficient

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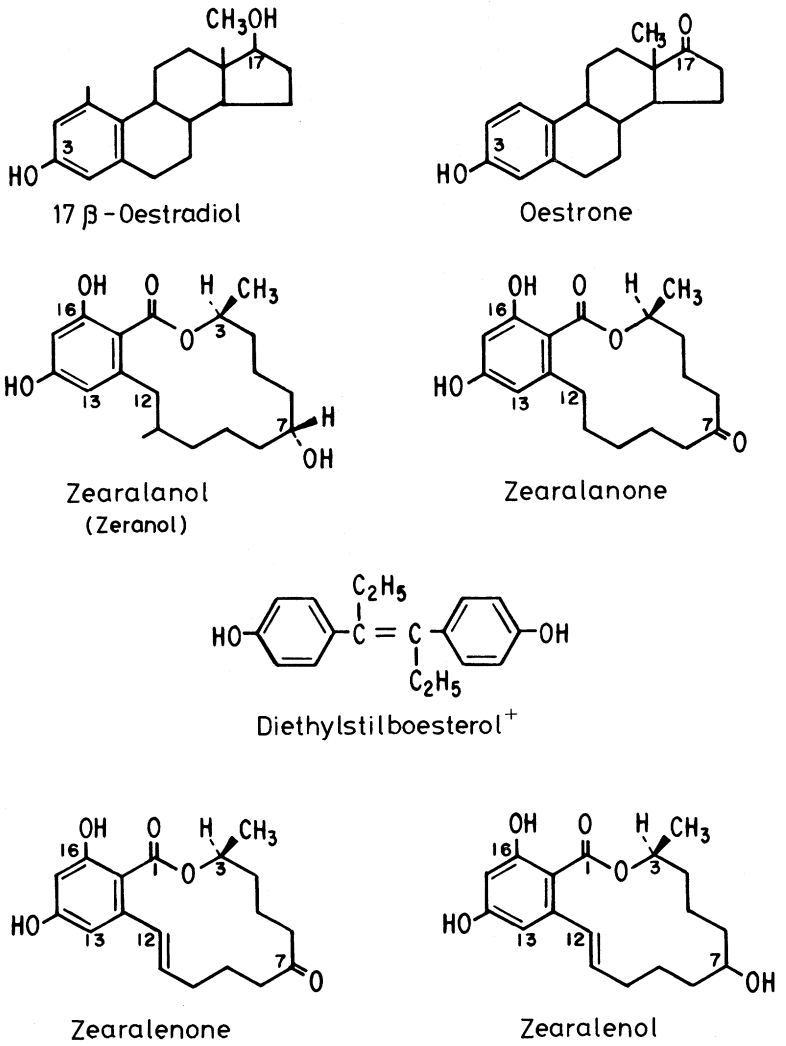

Figure 1. Structures of the anabolic oestrogens oestradiol, zeranol and DES; their metabolites, oestrone and zearalanone; and structurally related mycotoxins zearalenone and zearalenol.
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withdrawal period between treatment and slaughter of these animals may result in high levels of residues in the edible portion of the treated animals. On the other hand, consumption of feeds contaminated with the oestrogenic mycotoxins zearalenone and zearalenol (Mirocha and Christensen 1974, Mirocha *et al.* 1979) may also leave residues in tissues of food producing animals. Long term consumption of foods containing endogenous hormonal residues above physiological levels in animals or residues of exogenous compounds with hormone-like action (Hidy *et al.* 1977, Kiang *et al.* 1978) may cause adverse health effects. Therefore a multi-residue screening method to detect these oestrogens is needed. The chemical structure of the oestrogens studied is shown in figure 1.

There are numerous techniques reported in the literature for analysis of these oestrogenic compounds and their metabolites. However, only some of those methods developed for analyzing fluids and tissues of food producing animals are

cited. Ryan (1976) reviewed the chromatographic methods for hormone residues in foods while the use of HPLC separation of oestrogens was summarized by Heftman and Hunter (1979). Radioimmunoassay (RIA) techniques for 'total oestrogens' in bovine and porcine fluids and tissues were reported by Henricks *et al.* (1971, 1983), Monk *et al.* (1975), Henricks (1976), Dunn *et al.* (1977), Henricks and Torrence (1977, 1978), and Hoffmann (1978), whereas Medina (1986) reported a direct RIA measurement of oestradiol in ether extracts of bovine serum. TLC or HPLC analysis of DES were reported by de Ruig *et al.* (1982a, 1982b) and Verbeke and Vankee (1983). RIA analysis for DES was reported by Benraad *et al.* (1981) and Gridley *et al.* (1983). James *et al.* (1982) and Trenholm *et al.* (1981) developed HPLC methods for zearalenol and zearalenone in urine, plasma, or liver tissue and a TLC method was reported by Howell and Taylor (1981). Chromatographic and RIA methods for detecting zeranol and its metabolites were reviewed by Baldwin *et al.* (1983). Zeranol residue was detected by RIA at low ppb in cattle and sheep urine up to 70 days after implantation (Dixon and Russell 1983). Multi-residue TLC analyses of the anabolics were published by Günther (1978), Wortberg *et al.* (1978), Verbeke (1979), de Ruig (1982), and also reported by Medina *et al.* (1983).

The instrumental methods offer a wide spectrum of analysis. Most chemical and instrumental methods are tedious, lack sensitivity and are more expensive requiring technical skill. Immunoassays are highly sensitive with detection levels of <100 pg, but analysis is limited to one specific compound against which the antibody was produced and non-specific binding of a steroid antibody tend to produce false positive results particularly with the use of polyclonal antibody. TLC methods are rapid, inexpensive, and suitable for screening compounds prior to analysis by either immunoassay, HPLC, GC-MS, or other techniques.

The objectives of our study were to develop a simple and rapid TLC procedure to resolve the anabolic oestrogens and their metabolites that possess high oestrogenic activities; to determine the feasibility of utilizing our previously developed alumina-ion-exchange method to clean-up tissue samples prior to TLC analysis; and to provide a noninstrumental rapid screening procedure for analysis of the anabolic oestrogens in tissues of food-producing animals.

Experimental

Reagents and equipment

Acetone, hexane, methylene chloride, methanol, and 2-propanol (all glass distilled) were obtained from Burdick and Jackson, Muskegon, MI; basic alumina (80-200 mesh, Brockman Activity I), iodine, toluene, sea sand, polyethylene transfer jumbo pipette (7·5 mL), and wide mouth bottle (4 oz) with screw cap; IEC Centra-R7 Centrifuge; Ag-MP-1 ion exchange resin from Bio-Rad, Richmond, CA; soluble starch from J. T. Baker Company, Phillipsburg, NJ; dimethyldichlorosilane, 17β-oestradiol, oestrone, zearalenol, and zearalenone from Sigma Chemicals, St. Louis, MO; acetic acid (gold label) and diethylstilboesterol from Aldrich Chemical Company, Milwaukee, WI; zearalanol and zearalanone from International Minerals and Chemicals Corporation, Terre Haute, IN; polypropylene pipette tips (5 ml) from Rainin Instrument Company, Woburn, MA; 2, 4, 6, 7 ^{3}H-oestradiol, 17-β, and Liquifluor from New England Nuclear, Albany, MA; silica gel plates (2·5×10 cm), HETLC (HL) from Analtech, Newark, DE; 5×20 cm

LK5D silica gel plate from Whatman, Clifton, NJ; Polytron from Brinkman, Long Island, NY; and sonicator from Heat Systems, Long Island, NY.

Preparation of standards

Stock solutions were made to contain 1 mg oestrogens per ml methanol and stored below 0°C in vials treated with 2% dimethyl-dichlorosilane in toluene. Working dilutions of 2·5 ng/ml or 10 ng/ml were prepared with hexane/methanol/2-propanol (80:15:5).

TLC analysis

Wide mouth jars (4 oz, 112 ml) with screw caps were used as developing tanks for the 2·5×10 cm plates. Ten ml of developing solvent (methylene chloride/methanol/2-propanol, 97:1:2 v/v) were placed in jars and allowed to equilibrate for 15 min. The plates (previously washed with 50:50 methanol: methylene chloride (v/v) and activated at 110°C for 20 min) were spotted and air-dried using a hair dryer. The plates were equilibrated about 15 min with the solvent by placing the plate on the dry part of a tilted jar (figure 2a). The jar was then placed upright for development (figure 2b). The macro plates (5×20 cm) were developed in cylindrical tanks using 20 ml developing solvent after a 15 min equilibration. Table 1 summarizes the conditions used in the analysis of anabolic oestrogens. After development, the plates were dried in an 85°C oven for 5 min and exposed to iodine vapors for 3-5 min. The background iodine was allowed to volatilize for 30 seconds

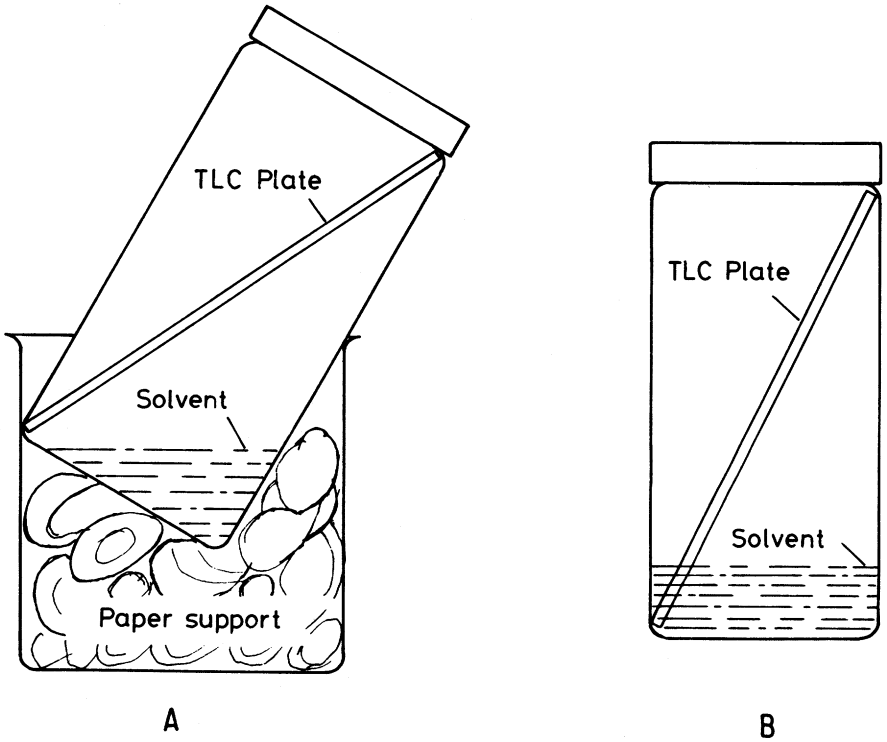

Figure 2. Set up for equilibration and development of micro-TLC plates. (A) equilibration position; (B) development position.

Table 1. TLC conditions used in the analysis of anabolic oestrogens. Developing solvent: methylene chloride/methanol/2-propanol (97:1:2). Visualizing agent: I_2 Starch.

			Development	
Gel type	Plate size (cm)	Gel thickness (μm)	Min dev. time (temp.)	Distance (cm)
Silica	2·5 × 10	250	4·5 (RT) 5·5 (4 °C)	6·5
Silica	5 × 20	250	30·0 (RT)	12·0
HETLC (Silica, HL)	2·5 × 10	150	9·0 (4 °C)	8·5

and the spots were revealed by spraying with 1% aqueous gelatinized starch solution. The starch suspension was heated at >70°C.

Sample extraction and purification

Chicken parts (thigh, drumstick, breast, liver, and adipose tissues) were analyzed separately. These chicken parts were homogenized separately in a Waring blendor and then stored at −20°C. Oestrogens were extracted from 1·0 gm muscle tissue by homogenizing in 5 ml acetone:water (95:5 v/v) using the Polytron for 2 min. The Polytron tip was rinsed with another 5 ml solvent, and sample was sonicated further for 5 min using 75% pulsed power. Liver and fat tissues (1 gm each) were homogenized by sonicating for 5 min. The samples were then allowed to stand for at least 30 min and centrifuged for 10 min at 3200 rpm. Each of the supernates was decanted and the pellet was extracted with another 5 ml of solvent, sonicated for 5 min, centrifuged and the supernates from each chicken parts were pooled. The volume of the pooled acetone extract was made up to 10 ml.

Aliquots (5 ml) of each pooled extract (except 2·5 ml for the liver extract) were applied to 1·5 g basic alumina column placed in tandem with the ion exchange column which contained 4 ml of Ag-MP-1 resin suspension in the phosphate form (Medina and Schwartz 1986). The extracts were allowed to percolate by gravity from the alumina column into the ion-exchange column. After washing the alumina column with 4 × 1 ml acetone:water (95:5 v/v), the alumina column was removed and the wall of the ion-exchange column was rinsed with 1 ml acetone:water solvent. The oestrogens were then eluted with 4 × 1 ml (acetic acid:acetone, 10:90 v/v). The acetic acid:acetone effluent was collected in silanized conical tube and evaporated to dryness with nitrogen. Distilled water (0·5 ml) was added into the tube and mixed vigorously to extract water soluble contaminants. The oestrogens in the aqueous mixture were extracted twice with 2 ml ether by using the IKA shaker for 5 min. The aqueous phase was separated by 'snap freezing' in dry ice/acetone. The ether extracts from each chicken part were pooled in silanized vials and dried in a stream of nitrogen. The walls of the vials were rinsed with 200 μl methanol and the solvent was evaporated off with nitrogen. The residue reconstituted with 50 μl hexane/methanol/2-propanol (80:15:5 v/v). One or 2 μl were applied onto HETLC plates (2·5 × 10 cm). All analysis were carried out in duplicate.

Analysis of fortified samples were carried out in three stages, i.e. increasing size of samples extracted and simultaneously decreasing amounts of test oestrogens

Table 2. Parameters used in extraction, purification and HETLC analysis of chicken muscle tissues.

Tissue sample size	Ml 95% acetone[a]	Oestrogen added[b]	(ng/g sample)	Reconstitution volume (μl)	ng analyzed by TLC
1 g	5,5	E_2, Za_2, DES, E_1, Z_1	250/0·5	100	5
2·5 g	10,8	E_2, Za_2	250/2·5	50	10
5·0 g	10,8	E_2, Za_2, DES, Z_1	50/5·0	25	4
		DES	100/5·0	25	8

[a] Tissues were extracted 2 times using volumes below.
[b] E_2 (oestradiol), Za_2 (zeranol), DES (diethylstilboesterol), E_1 (oestrone), Z_1 (zearalenone).

and volume of solvent used to reconstitute residues (table 2). Aliquots of 1 g tissues were extracted as described in previous paragraphs. Extracts of 0·5 g tissue equivalent were fortified with 250 ng each of the five oestrogen standards (17β-oestradiol, oestrone, DES, zeranol, zearalenone), and subsequently passed through the alumina/ion-exchange columns, washed and eluted with 10% acetic acid in acetone and dried with nitrogen gas. The residues were reconstituted with 100 μl of hexane/methanol/2-propanol (80 : 15 : 15) to contain 2·5 μg of each standard per μl. Two microliters were applied to the micro-HETLC plates (2·5 × 10 cm). A 2·5 g portion of chicken muscle tissue was also extracted as described in the preceding paragraph but using 10 and 8 ml for the first and second extractions, respectively. The extracts were then fortified with 250 ng each of binary mixture of oestradiol and zeranol and subsequently cleaned up through the basic alumina and ion-exchange columns. The residues were reconstituted with 50 μl hexane/methanol/2-propanol. Two microliters containing 10 ng each of the fortified estrogens were spotted on the plates. Aliquots of 5 g chicken muscle tissues were also extracted with 10 and 8 ml of 95% acetone. The acetone extracts were fortified with (1) 50 ng estradiol and DES, (2) 50 ng oestradiol and zearalenone, (3) 50 ng each of oestradiol and zeranol and (4) 50 ng oestradiol and 100 ng DES. The fortified extracts were purified through the dual columns and the dried residues were reconstituted with 25 ml hexane/methanol/2-propanol. HETLC plates were spotted with 2 μl samples and standards containing 10 μg/μl of estradiol, zearalenone, DES and zeranol were also spotted alongside the samples, spacing them approximately 0·8 cm apart. All samples were analyzed twice by HETLC.

The detectable quantity in TLC analysis was estimated by calculating the theoretical amount of spiked oestrogens spotted on the plates and comparing the intensity of bands of the known amount of standards versus the intensity of bands of samples.

Recovery Measurements

Purification recoveries were monitored by adding a known amount of ^{3}H-oestradiol to the extracts prior to the clean-up process. Radioactivity loss from sample was measured in the acetone:water wash which was collected in scintillation vials, pooled, dried under nitrogen and counted in the scintillation counter after addition of 10 ml scintillation fluid. Recovery was directly measured by purifying a set of samples spiked with ^{3}H-oestradiol in separate columns applying the same conditions used for samples purified for TLC analysis. Radioactivity content of the

of the acetic acid effluent or of the ether extract was counted for recovery and the acetone/water wash was also counted for loss in the purification process.

Results and discussion

The mean R_f values of oestradiol, oestrone, *trans*-DES, *cis*-DES, zearalenone, and zeranol, when chromatographed individually or as mixtures at room temperature are shown in figure 3A and 3B. The micro TLC plates (2·5 × 10 cm) in figure 3A

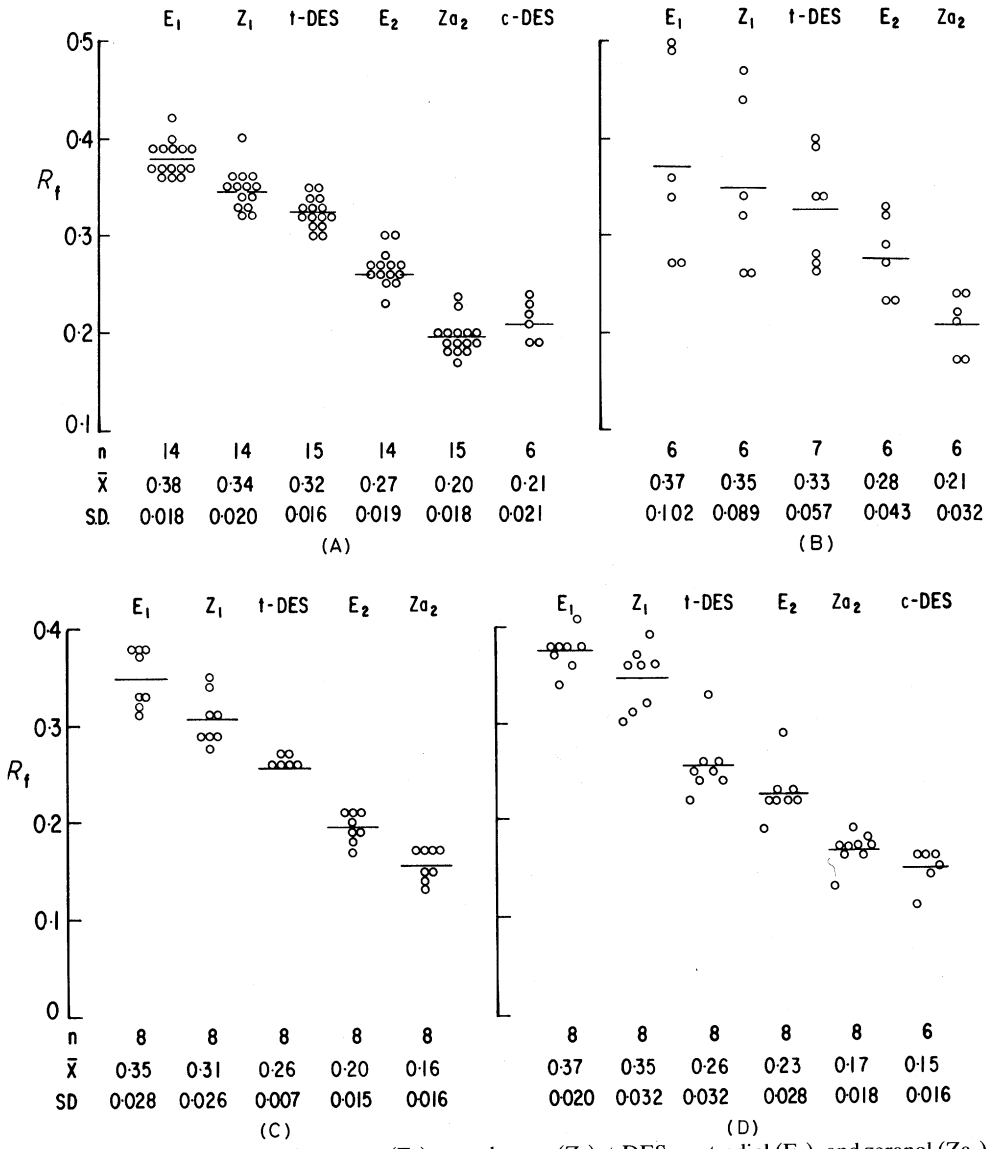

Figure 3. Composite plots of oestrone (E_1), zearalenone (Z_1), t-DES, oestradiol (E_2), and zeranol (Za_2) analyzed as single compound or as a multi-component mixture at various conditions.
n = number of analyses; $\bar{X}$ = mean R_f represented by — in figure; o = R_f of each oestrogen; S.D. = standard deviation. (A) 2·5 × 10 cm unbonded silica gel plate developed at room temperature, (B) 5 × 20 cm bonded silica gel plate at room temperature; and (C) 2·5 × 10 cm unbonded silica gel developed at 4°C and (D) 2·5 × 10 cm HETLC-HL silica gel developed at 4°C.

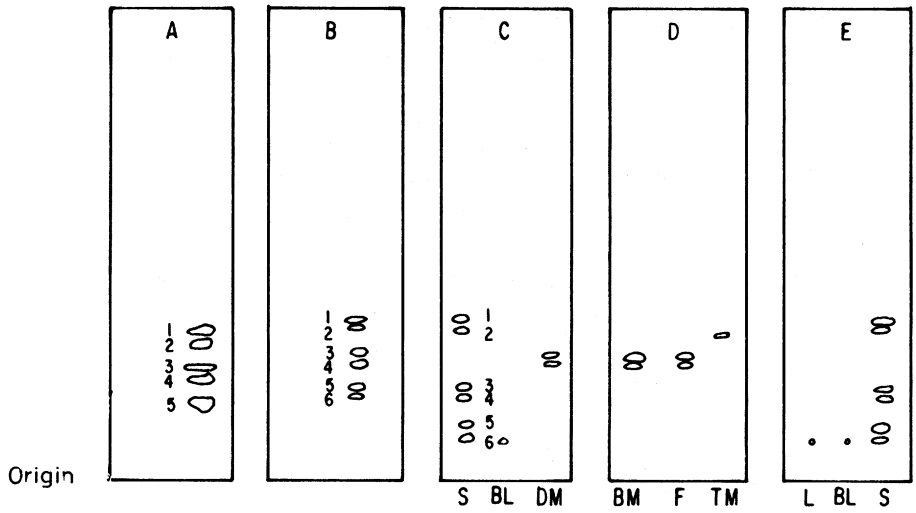

Figure 4. Thin layer chromatograms of anabolic oestrogens.
(A) 5 component standards containing estrone (1), zearalenone (2), t-DES (3), oestradiol (4), zeranol (5) in increasing order of polarity analyzed on unbonded 2·5 × 10 cm TLC plate; (B) 7-component standards containing 20 ng each of oestrone (1), zearalenone and zearalanone (2), t-DES (3), oestradiol (4), zeranol and zearalenol (5) and c-DES' (6); (C and E) 5 component standards at lane S, blank column effluent (BL), column purified extract of 0·5 g drumstick muscle extract (DM); (D and E) column purified extracts of 0·5 g breast muscle (BM), fat (F) thigh muscle (TM) and liver (L). Bonded HETLC were used in (B)–(E).

showed good reproducibility, while the use of the macroplates (5 × 20 cm) in Figure 3B resulted in some scatter, but better resolution was achieved. The separation of the five oestrogens was improved when the plates were developed at 4 °C (figure 3C). The use of HETLC (figure 3D) also yielded better resolution than the regular silica gel plate (figures 3A and 3C). Spotting an unbound silica gel plate created a hole on the plate and when dried by forced air, the silica gel was blown off the plate carrying some of the applied sample with it resulting in sample loss. The use of bonded HETLC eliminated this problem. Metabolites of zearalenone and zeranol (zearalenol and zearalanone, respectively) were also analyzed along with five other oestrogens. Results showed six discrete bands (figure 4B). The sixth and slowest moving band is *cis*-DES, isomerized from *trans*-DES. Upon dissolution and storage in solvent, *trans*-DES partly isomerized into *cis*-DES, and if stored in solvent below 0 °C this isomerization was somewhat retarded. Zearalanone and zearalenol did not separate from either zearalenone or zeranol and had identical R_f values when chromatographed as individual or mixed compounds. This result suggests that occurrence of high quantities of zearalenol in samples can interfere with the analysis of zeranol. Mirocha and co-workers (1979) reported occurrence of zearalenol in amounts of 0·15–4·0 μg per gram of animal feed. However, the mycotoxins and the drug can be resolved by normal or reversed phase HPLC (Medina *et al.* 1985, Medina and Sherman 1986), and their structures can be confirmed by mass spectroscopy. When standards were analyzed, the minimum detectable amounts were 2·5 ng on micro TLC or HETLC plates and 5 ng on 5 × 10 cm plates. Minimum detectable amounts of zeranol and estradiol were 2–3 times higher than estrone, zearalenone, or DES.

The sensitivity and resolution of our HETLC procedure was compared with

Table 3. Comparative R_f values of anabolic oestrogens.

	$CHCl_3/C_2H_5OH$/benzene (36:1:4)		CH_2Cl_2/CH_3OH/2-prop. (97:1:2)
Oestrone	0·39[a]	0·34[b]	0·38[b]
Zearalenone	0·30	0·29	0·35
Zearalanone	[c]	0·29	0·35
t-DES	0·22	0·21	0·26
Oestradiol, 17-β	0·22	0·21	0·23
Zearalenol	[c]	0·15	0·17
Zearalanol	0·18	0·15	0·17
c-DES	0·08	0·09	0·15

[a] Reported by Verbeke (1979).
[b] Chromatographed in our laboratory using HETLC and visualized with I_2-starch.
[c] No reported analysis.

the technique described by Verbeke (1979) using chloroform/ethanol/benzene (36:1:4 v/v) as developing solvent and visualizing the spots by charring with H_2SO_4 or fluorescence activation with acetic anhydride and H_2SO_4. Results showed that methylene chloride/methanol/2-propanol had better resolution, separating estradiol from t-DES (table 3), and visualization of the spots with iodine and starch was more sensitive than the methods described by Verbeke and co-workers (1979) However, chromophores formed by I_2-starch are not stable and last only for a few minutes but can be stabilized by storing plates below 0°C, wrapped in polyethylene film.

Extracts of thigh, drumstick, breast, liver and adipose tissue previously cleaned-up through the alumina and ion-exchange columns and analyzed separately by HETLC at 4°C did not give bands that interfered with the five oestrogen standards (Figures 4C, 4D and 4E). Fortifying the extracts of breast or drumstick tissue at 500 ppb (250 ng each of oestrogen added to 0·5 gm tissue extract), followed by the described purification procedure and HETLC analysis resulted in distinct spots with R_fs of 0·25, 0·24, 0·19 for DES, oestradiol, and zeranol respectively versus standard R_fs of 0·26, 0·23 and 0·17. Application of 2-μl sample on the plate was calculated to theoretically contain 5 ng of each component. Zearalenone and oestrone were not detectable under these conditions but can be detected at higher concentration. In this analysis a four year old resin was used for purification. However, in later studies, new batches of AgMP-1 were used for the clean-up process. The effluents from several lots of new resin showed artifacts at R_fs where DES, zearalenone, zearalanone or oestrone migrated. The earlier procedure was modified to remove water soluble artifacts in the HOAc effluent by dissolving them in distilled water and extracting the oestrogens with ether. Analysis of chicken muscle extract fortified with oestradiol at 200 ppb was a deep blue band with R_f 0·43 versus standard oestradiol R_f of 0·42 when the theoretical equivalent of 50 ng was spotted and visualized with starch and iodine. A binary mixture of zeranol and oestradiol (100 ppb each) fortified in chicken muscle extracts were also clearly detected when the theoretical equivalent of 10 ng was chromatographed. R_fs of zeranol and oestradiol in samples were 0·22 and 0·27, respectively versus 0·22 and 0·28 in standards. Intensity of the sample bands were comparable to the intensity of a 10 ng standard spotted in the same plate suggesting a quantitative recovery of the compounds used in fortification. In the third stage of analysis when samples were

Table 4. HETLC analysis of purified chicken muscle tissue extracts fortified with mixtures of estradiol, DES, zearalenone or zeranol.

	Oestrogens	Amount added (ng)	ppb level	ng Analyzed	Results
(1)	Oestradiol	50	10	4	+
	DES	50	10	4	+
(2)	Oestradiol	50	10	4	+
	Zearalenone	50	10	4	?[a]
(3)	Oestradiol	50	10	4	+
	Zeranol	50	10	4	+
(4)	Oestradiol	50	10	4	+
	DES	100	20	8	++

[a] The zeralenone band could not be distinguished from an interfering component of chicken muscle also shown in unfortified sample.

Table 5. R_f values of standard anabolic oestrogens versus oestrogens isolated from fortified chicken muscle tissue extracts. Samples were fortified with binary or ternary mixtures of oestrogens and chromatographed alongisde standard mixture of zearalenone, oestradiol, DES and zeranol. Samples and standards containing 4 ng and 10 ng oestrogens respectively were spotted onto TLC plates.

		R_f	
	Oestrogens	Standard	Sample
(1)	Oestradiol	0·35	0·35
(2)	Oestradiol + DES	0·29	0·28
		0·34	0·34
(3)	Oestradiol + DES	0·29	0·29
		0·36	0·35
(4)	Zearalenone + Zeranol	0·45	0·45[a]
		0·22	0·21
(5)	Zearalenone + DES	0·45	0·44[a]
		0·37	0·38
(6)	Zearalenone	0·44	0·44[a]
	Oestradiol	0·31	0·31
	Zeranol	0·24	0·25
(7)	DES	0·36	0·35
	Estradiol	0·29	0·29

[a] An interfering band from tissue extracts also migrates at R_f 0·44–0·45.

fortified at 10 ppb levels (50 ng oestrogen/5 g sample) and theoretical equivalents of 4 ng test oestrogens were analyzed by HETLC, oestradiol, DES and zeranol were detected as positive spots (table 4). One sample containing 8 ng DES/2 µl showed a more intense spot than 4 ng DES band. Zearalenone was not detectable at 4 ng when oestrogens were visualized with I_2-starch due to an interfering band present in column purified chicken muscle extract. Table 5 shows R_f values of anabolic oestrogens isolated from muscle tissue extracts analyzed by HETLC. High accuracy and precision is shown by $\pm 0{\cdot}01$ deviation of sample R_fs from corresponding oestrogen standards. The use of oestradiol as fortification component also served as internal standard. Oestradiol is stable in both the extraction and purification processes and the zone where it migrates on TLC has no interferences from sample or column artifacts. This non-instrumental analysis of anabolic oestrogens was

developed for rapid screening. Should there be positive bands indicating presence of oestrogens, other instrumental technique such as HPLC or GC-MS must be applied to confirm the results of TLC analysis.

Column recoveries of fortified extracts were 95·6% (SD, 2·2; $n=30$) as determined by fortifying separate sets of samples with ^{3}H-oestradiol, passing the extracts through the columns and measuring the radioactivity of column washes. Thirty columns were monitored in 15 experiments. In five experiments, residual radioactivity remaining in the columns was eluted and showed a range of 0–3·4% of total radioactivity. Systematic studies of the accuracy and precision of this purification technique are discussed in our previous work (Medina and Schwartz 1986). It was not practical to measure the ^{3}H radioactivity on the HETLC plates using a surface scanner because counting efficiency of the scanner was only 20% of the scintillation counter. Furthermore, ^{3}H label seemed to break-off from oestradiol giving scattered radioactivity peaks. In addition to monitoring the radiotracer activity, colour intensity of the corresponding oestrogen bands in the samples was compared with known amounts of standards and both sample and standard seemed to agree quantitatively.

Tissues were not fortified directly, but instead, the acetone extracts were fortified to monitor recovery of oestrogens through the purification and TLC procedures. In this work, we also reported that extraction recovery of the tracer from fortified tissues ranged only from 65% to 75% even after exhaustive extraction of the tracer, i.e. sonication and centrifugation three times. This phenomenon was also observed by other investigators (Metzler *et al.* 1981; Gridley *et al.* 1983). The 'loss' of 25%–35% is apparently due to irreversible binding of the added oestrogens or tritium label with tissue components and remains in the aqueous residue which cannot be extracted by current techniques. When tissues are fortified with test oestrogens this loss should not be compensated in measured values as it might overestimate the amount of free oestrogens in samples analyzed. Therefore, results derived from this analysis can only be interpreted as extractable oestrogens.

In conclusion, the extraction, purification, and HETLC procedures described, provide a rapid, simple and inexpensive method to screen approximately 4 ng of oestradiol, DES and zeranol in chicken muscle tissue extracts containing oestrogens in low parts per billion (μg/kg). This method should be applicable to the screening of oestrogens in the edible tissues of cattle and sheep. Other visualization chemicals are being investigated to detect zearalenone, and zeranol at low nanogram levels.

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